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Thermal Denaturation of Whey Proteins in Skim Milk

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Heat-induced denaturation of whey proteins, analyzed by reversed-phase HPLC, indicated that denaturation proceeds in two stages. The first stage of denaturation proceeds less rapidly than the second stage. Aggregation of whey proteins begins at about 70 °C, and more aggregation was found to occur in sweet whey than in acid whey. The dimer of β-LG and the β-LG–α-LA complex were found to form preferentially in heated acid whey. Comparison of the second derivative Fourier transform infrared spectra of reconstituted skim milk and high-heat nonfat dry milk (NDM) powder (85 °C) showed that heat treatment results in formation of two new bands (1684 and 1613 cm^{-1}) in the amide I region at frequencies usually associated with intermolecularly hydrogen bonded β sheets. The bands were smaller in sweet whey, compared with acid whey; difference spectra indicated that β-LG was primarily responsible for the formation of heat-induced hydrogen bonding.

INTRODUCTION

Heat treatment of skim milk is used widely to modify the functional behavior of nonfat dry milk (NDM) in milk products. Preheat treatment of skim milk before spray drying is used as a means of improving water absorption and functional properties of NDM powders. High-heat powders (85 °C) are used in baking to improve extensibility of dough. These powders are unsuitable for cheese making because complexes formed render the powders resistant to rennet action; low-heat powders (63 °C) are more desirable. Heat-induced denaturation and interactions of whey proteins in skim milk have been studied in different milk protein systems under a variety of experimental conditions. The data have been reviewed with respect to physicochemical changes and functional properties (Kinsella, 1984; Mulvihill and Donovan, 1987; Kinsella and Whitehead, 1989).

β-Lactoglobulin (β-LG), the major protein in whey, has also been extensively characterized (Dalgleish, 1990; O'Neill and Kinsella, 1988; Harwalkar, 1980; Sawyer et al., 1971; Casal et al., 1988; Pipez et al., 1986; Byler and Purcell, 1989a,b). Although many studies have been conducted on the thermal denaturation of this protein, the mechanism of denaturation remains unclear. Specific rotation and Fourier transform infrared spectroscopy (FTIR) showed that heat denaturation of β-LG in aqueous solution occurs in two stages (Harwalkar, 1980; Sawyer et al., 1971; Casal et al., 1988). It is not clear, however, if β-LG is thermally denatured in whey in the same manner as in solution. In this study the effect of heat treatment on whey protein denaturation in skim milk was examined by liquid chromatography (LC) and FTIR to explain its behavior in food systems.

MATERIALS AND METHODS

Materials. α-Lactalbumin (α-LA) and β-LG AB were purchased from Sigma (St. Louis, MO).

Nonfat Dry Milk. Raw pooled herd milk from Holstein, Ayrshire, and Brown Swiss cows was skimmed at 38 °C and preheated at 63 and 85 °C for 30 min, concentrated, and then spray-dried to yield nonfat dry milk (NDM) powders according to a previously published procedure (Parris et al., 1989).

Whey. Acid whey was prepared by adjusting the pH of the skim milk to 4.6 with 1 N HCl. The samples were held at room temperature for 30 min and then filtered through S&S No. 605 filter paper. The resulting whey was dialyzed with stirring against raw skim milk or phosphate buffer (pH 6.8) as previously described (Parris et al., 1990; Morr and Josephson, 1968). Alternatively, sweet whey was prepared by adding 0.1 mL of rennet to 100 mL of skim milk at 32 °C. After 30 min, the curd was removed by passing the suspension through cheesecloth.

Milk Dialysate. Water was placed in a dialysis bag (8000-Da cut-off) and dialyzed against skim milk at a water to milk ratio of 1:100 at 4 °C for 72 h; the contents were removed from the bag and frozen until needed.

Heat Treatment. Reconstituted skim milk (10 g/100 mL), acid whey (0.51 g/100 mL), sweet whey (2.0 g/100 mL), or a mixture of 0.35 g β-LG AB and 0.13 g/100 mL α-lactalbumin (α-LA) was heated in a water bath at various temperatures for 30 min.

Whey Protein Denaturation. Heat denaturation of whey proteins was determined by reversed-phase HPLC (RP-HPLC) as previously reported (Parris and Baginski, 1991). Using this technique, the extent of protein denaturation was determined by comparing the sum of the normalized absorbance peaks of whey proteins eluted from the RP-HPLC column for heated milk samples to those of an unheated control skimmed at 38 °C. The column used was a 0.46 × 25 cm C-4 reversed-phase, 10-μm particle size, Vydac (Hesperia, CA). The mobile phase was solvent A [0.1% trifluoroacetic acid (TFA)] and solvent B (acetonitrile, 0.1% TFA). Whey protein aggregate, dimer, and complex formation on heating was determined by size exclusion chromatography (SEC) as described elsewhere (Van Boekel and Walstra, 1989). Proteins were separated on a Zorbax GF-250 column (Du Pont, Wilmington, DE) using a buffer consisting of 0.25 M phosphate, pH 6.7, at 0.8 mL/min. Samples were filtered through a 0.45-μM filter before injection onto the column.

Gel Electrophoresis. Polyacrylamide gel electrophoresis of milk proteins was carried out on a Phast System (Pharmacia, Piscataway, NJ) using a 8–25% gradient acrylamide gel as described in Parris et al. (1990).

Infrared Spectroscopy. Samples were prepared as solutions in 50 mM potassium monobasic phosphate-*d* buffer (pD 6.2) made with D_2O. pD was calculated by adding 0.4 to the pH measured with a glass electrode (Covington et al., 1968). The concentrations of the samples were 10% w/v for NDM, 5.2% w/v for acid whey, and 3.5% w/v for β-LG A/B. Small portions of each solution were placed in 75 μm path length IR cells with Teflon spacers between CaF_2 windows. Spectra were collected at ambient temperature using a Nicolet 740 SX FTIR spectrometer with a water-cooled Globar source, a Ge-coated KBr beam splitter, and a broad-band MCT detector. All spectra were recorded at a nominal resolution of 2 cm^{-1}. Data collection was terminated after co-adding 4096 double-sided interferograms (0.44 s/scan). Each interferogram was converted into an absorption spectrum after the application of phase correction,

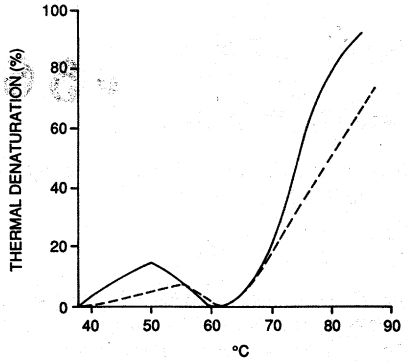

Figure 1. Typical whey protein denaturation profile for heat-treated skim milk (—) and acid whey (- - -) samples.

Happ–Genzel apodization, and a fast Fourier transform algorithm. The spectrometer and sample chamber were purged continuously with dry nitrogen gas. Spectral contributions from uncompensated H_2O vapor in the light path and from the buffer were subtracted using programs provided with the Nicolet FTIR software, version 4.3. Factors for water vapor subtraction were determined by subtracting a second-derivative spectrum of water vapor from the second-derivative spectrum of the sample. Differentiation of an observed spectrum was performed with a program provided with Nicolet software as described elsewhere (Susi and Byler, 1983; Byler and Susi, 1986) except that second-derivative spectra were obtained by applying DR1, first-derivative function, twice. Absorbances for all DR2 spectra shown in the figures were plotted to the same scale.

RESULTS AND DISCUSSION

Changes in milk protein profiles in NDM powder prepared from heat-treated skim milk have been identified using RP-HPLC (Parris et al., 1990). Whey proteins in heated acid whey did not denature to the same extent as whey proteins in heated skim milk (Figure 1). At 85 °C approximately 95% of the whey proteins in skim milk are denatured compared to about 70% in acid whey. In addition, whey proteins prepared from the same batch of milk exhibit the same denaturation minima (Figure 1). These minima ranged from 61 to 67 °C with different batches of milk. The temperature at which the minima occurred corresponded to the formation of a whey–casein complex in skim milk and a whey complex in acid whey by RP-HPLC and gel electrophoresis. The minima could be due to conformational changes in the whey protein with greater exposure of aromatic residues before complex formation (Parris and Baginski, 1991). Whey proteins appear to denature more rapidly after the minima for both skim milk and acid whey. Harwalkar (1980) reported that heat denaturation of β-LG AB in solution at pH 2.5 occurs in two stages; the first proceeds relatively slowly and is reversible, while the second stage is rapid and irreversible. Sawyer et al. (1971) reported similar thermal denaturation kinetics for β-LG at pH 7.0.

Gel electrophoresis and SEC indicate that whey proteins begin to aggregate at about 70 °C by the formation of high molecular weight material that did not enter the SDS–PAGE running gel and the appearance of an early eluting peak by SEC. Comparison of the effect of heat treatment on acid whey, sweet whey, and a mixture of β-LG AB and α-LA in milk dialysate indicated that whey proteins do not aggregate to the same extent on heating (Table I). More protein aggregation occurred in sweet whey and the β-LG/α-LA mixture than in acid whey at elevated temperatures. Slightly more aggregates were formed in acid whey which was dialyzed against skim milk. SDS–PAGE indicated the formation in acid whey of a significant amount of a dimer of β-LG and a β-LG—α-LA complex having molecular weights of 36 000 and 33 500, respectively. Formation of denatured dimers of whey proteins on heating have been proposed (Hill, 1988; Garrett et al., 1988). Xiong and Kinsella (1990) reported the formation of dimers of β-LG and α-LA and the β-LG—α-LA complex in whey protein isolate incubated in 6 M urea at pH 7.0 and 25 °C.

Table I. Percentage Distribution of Protein Components in Whey after Heat Treatment

	temp, °C				
component	38	70	75	80	85
acid whey[a]					
aggregates	0	0	2	4	5
dimer and complex	0	1	5	15	25
β-LG	55	49	39	23	10
α-LA	45	50	54	59	60
acid whey[b]					
aggregates	0	0	1	2	12
dimer and complex	0	3	4	7	11
β-LG	56	54	44	34	12
α-LA	44	43	51	57	64
sweet whey					
aggregates	0	1	2	23	57
dimer and complex	0	2	4	4	4
β-LG	58	54	52	39	16
α-LA	42	43	43	34	23
mixture[c]					
aggregate	0	1	21	54	78
dimer and complex	0	0	0	0	0
β-LG	55	49	41	22	7
α-LA	45	50	38	24	15

[a] Dialyzed against phosphate buffer. [b] Dialyzed against skim milk. [c] Mixture of β-LG AB and α-LA in dialysate.

Heat-induced aggregation may occur less readily in acid whey because α-LA does not interact with the denatured or polymerized form of β-LG. At the higher temperatures undenatured α-LA represents approximately 60% of the protein components in acid whey. Matsudomi (1991) found that lysozyme, a structural homologue of α-LA, interacted more with the monomeric molecule of the fully unfolded ovalbumin heated to its thermal transition temperature for denaturation than with either the native or thermally polymerized molecule.

Using infrared spectroscopy, Casal et al. (1988) observed that the thermal denaturation of β-LG B occurs in two stages and that the first stage is completed at about 60 °C. Conformational changes of thermally denatured whey proteins, reconstituted β-LG AB, acid whey, skim milk, and NDM powders from heated skim milk were investigated with FTIR at room temperature. FTIR permitted more detailed studies of the secondary structure of the milk proteins with better wavenumber accuracy and reproducibility. Absorption spectra for solutions of reconstituted skim milk (38 °C) and high-heat NDM powder (85 °C), however, were found to be very similar to spectra of casein and to have few differences between them (Figure 2). Differences between these spectra were observed by applying a mathematical resolution and enhancement technique, viz., second-derivative (DR2) spectroscopy.

The DR2 spectra of the above samples showed the effect of thermal denaturation of milk proteins, particularly in the amide I region, 1700–1620 cm^{-1} (compare parts a and b of Figure 3). Absorption in the amide I region arises mainly from complex vibrations in the peptide bonds in the backbone of the protein chain (Byler and Susi, 1986). The major portion of these complex vibrations is a carbonyl stretch vibration. Spectral interpretation is best understood for the amide I region which is the most useful for monitoring conformational changes. Five major bands in the amide I region of skim milk were no longer present

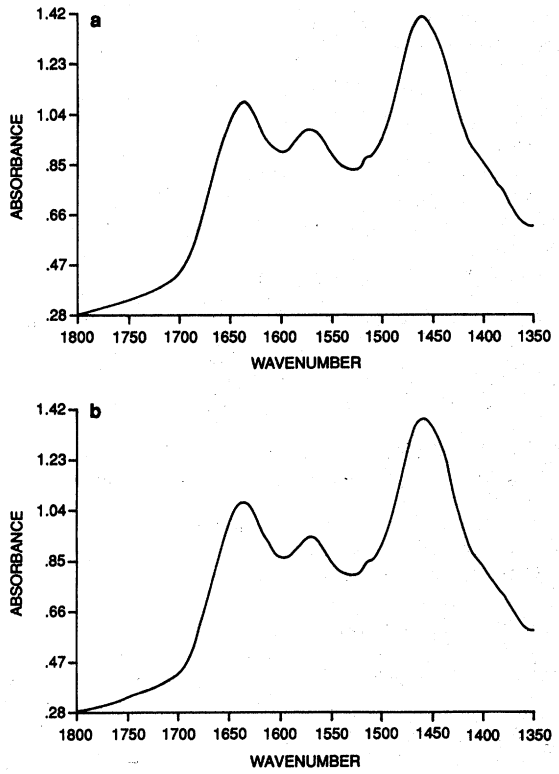

Figure 2. Infrared spectra of the amide I and amide II region for reconstituted milk samples. (a) Lyophilized milk skimmed at 38 °C; (b) high-heat NDM powder, 85 °C.

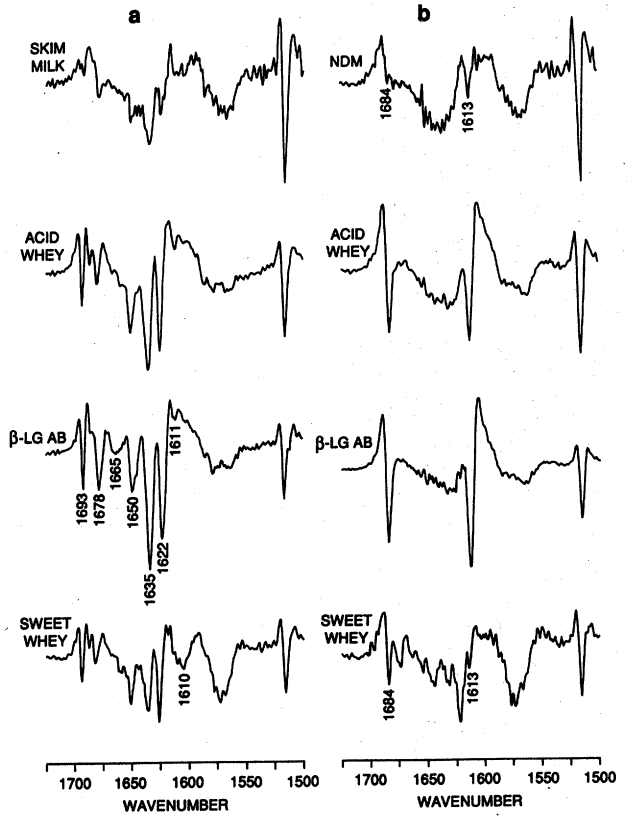

Figure 3. DR2 FTIR spectra of milk samples in Figure 2, acid whey, β-LG AB, and sweet whey. (a) 38 °C; (b) 85 °C.

in the heated NDM (85 °C) powder (Figure 3b). Two new bands (1684 and 1613 cm^{-1}) were present in this region (discussed in greater detail below). Since spectra of casein solutions under the same conditions do not change in the amide I region as the temperature increases to 95 °C (Byler and Purcell, 1989b), the changes observed when skim milk

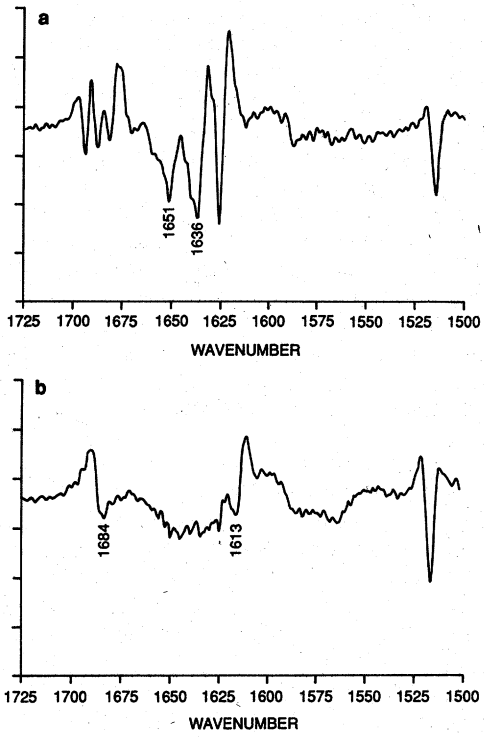

Figure 4. Difference spectra of acid whey minus β-LG AB. (a) 38 °C; (b) 85 °C.

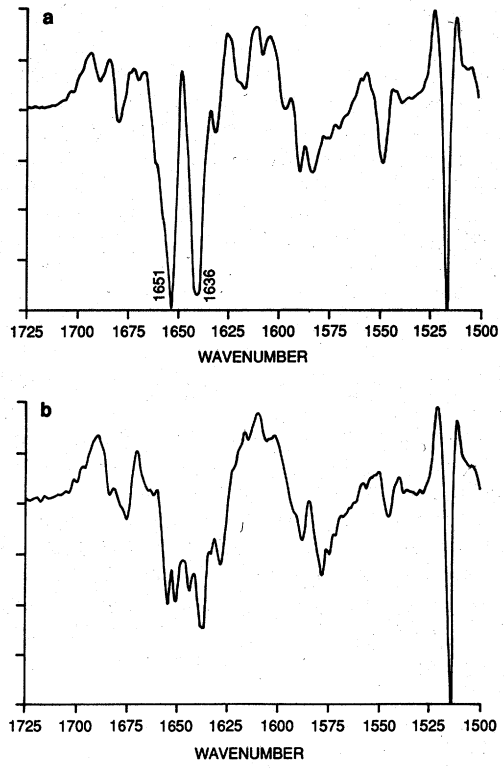

Figure 5. DR2 FTIR spectra of α-LA. (a) In 20 mM Ca^{2+}; (b) depleted of Ca^{2+}.

is heated are due to whey proteins. Moreover, spray-drying does not significantly denature whey proteins, as no apparent change was observed in the DR2 spectra of reconstituted skim milk and low-heat NDM (63 °C) powder (not shown).

The DR2 spectrum of acid whey exhibits at least five amide I component bands and closely resembles that of β-LG AB (Figure 3a). The structure of crystalline bovine β-LG B reported by Papiz et al. (1986) indicates that it consists of antiparallel β sheets, α helix, and unordered

strands. Assignment of the seven bands in the amide I region for β-LG is based on previous studies of proteins by vibrational spectroscopy (Casal, 1988; Byler and Purcell, 1989a,b). The three bands at 1678, 1635, and 1622 cm^{-1} (Figure 3a) correspond to antiparallel β sheets, the broad band at 1665 cm^{-1} is due to turns, and the band at 1650 cm^{-1} represents the overlap of signals from α helix and unordered segments. The band at 1693 cm^{-1} can be assigned to protonated carboxylate groups, while the band at 1611 cm^{-1} originates from the C=C stretching vibration in aromatic side-chain residues (Casal et al., 1988).

The spectrum for heated acid whey changed dramatically with a number of unresolved bands in the amide I region and the appearance of the same bands at 1684 and 1613 cm^{-1} found in the reconstituted NDM (85 °C) powder (Figure 3b). The new bands are probably due to new intermolecularly hydrogen bonded β sheets, since they are also present in heated β-LG AB (Figure 3b) and are reported to appear prior to gelation (Byler and Purcell, 1989b). The spectrum for sweet whey at 38 °C is essentially the same as that of acid whey in the amide I region except for a group of bands centered around 1610 cm^{-1} which can be attributed to protonated carboxylate groups (Figure 3a). After heating, the two bands at 1684 and 1613 cm^{-1} were much less intense than in heated acid whey (Figure 3b), indicating that intermolecular hydrogen bonding was less extensive on heating. This could be a result of lactose-induced stabilization of protein–protein interactions during heat treatment.

Electronically subtracting the DR2 spectrum of β-LG from that of acid whey (38 °C) yielded a spectrum of α-LA and bovine serum albumin (BSA) (Figure 4a). The difference spectra at 85 °C contained very small bands at 1684 and 1613 cm^{-1}, indicating that β-LG is primarily responsible for forming the heat-induced intermolecular hydrogen bonds in whey (Figure 4b). The difference spectrum at 38 °C (Figure 4a) was similar to that of α-LA in 20 mM Ca^{2+} with similar bands at 1651 and 1636 cm^{-1} which can be attributed to α helix and β sheet (Figure 5a). These bands are not present in the spectrum for α-LA depleted of Ca^{2+} (Figure 5b), indicating that the Ca^{2+} is tightly bound to α-LA and is not removed by isoelectric precipitation and dialysis against phosphate buffer.

In conclusion, RP-HPLC and FTIR indicate that whey proteins are thermally denatured in two separate stages with protein aggregation occurring in the second stage. More heat-induced aggregation occurs in sweet than acid whey, even though less intermolecular hydrogen bonding occurs in sweet whey. This seems to indicate that protein aggregation in sweet whey can also be attributed to less specific hydrophobic interactions and/or calcium-dependent linkages. Such information should be useful in explaining the functional behavior of heat-treated skim milk in food systems.

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